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(54) **Increased production of thermus aquaticus DNA polymerase in E. coli**

Erhöhte Produktion von thermus aquaticus DNA Polymerase in E. coli

Augmentation de la production d'ADN de thermus aquaticus polymerase chez E. coli

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Description

This invention relates to the field of genetic engineering. More particularly, this invention relates to the alteration of a native gene to provide a mutant form having improved expression in E. coli.

One of the major achievements in recombinant technology is the high-level expression (overproduction) of foreign proteins in procaryotic cells such as Escherichia coli (E. coli). In recent years, this technology has improved the availability of medically and scientifically important proteins, several of which are already available for clinical therapy and scientific research. Overproduction of protein in procaryotic cells is demonstrated by directly measuring the activity of the enzyme with a suitable substrate or by measuring the physical amount of specific protein produced. High levels of protein production can be achieved by improving expression of the gene encoding the protein. An important aspect of gene expression is efficiency in translating the nucleotide sequence encoding the protein. There is much interest in improving the production of bacterial enzymes that are useful reagents in nucleic acid biochemistry itself, for example, DNA ligase, DNA polymerase, and so forth.

Unfortunately, this technology does not always provide high protein yields. One cause of low protein yield, is inefficient translation of the nucleotide sequences encoding the foreign protein. Amplification of protein yields depends, inter alia, upon ensuring efficient translation.

Through extensive studies in several laboratories, it is now recognized that the nucleotide sequence at the N-terminus-encoding region of a gene is one of the factors strongly influencing translation efficiency. It is also recognized that alteration of the codons at the beginning of the gene can overcome poor translation. One strategy is to redesign the first portion of the coding sequence without altering the amino acid sequence of the encoded protein, by using the known degeneracy of the genetic code to alter codon selection.

However, the studies do not predict, teach, or give guidance as to which bases are important or which sequences should be altered for a particular protein. Hence, the researcher must adopt an essentially empirical approach when he attempts to optimize protein production by employing these recombinant techniques.

An empirical approach is laborious. Generally, a variety of synthetic oligonucleotides including all the potential codons for the correct amino acid sequence is substituted at the N-terminus encoding region. A variety of methods can then be employed to select or screen for one oligonucleotide which gives high expression levels. Another approach is to obtain a series of derivatives by random mutagenesis of the original sequence. Extensive screening methods will hopefully yield a clone with high expression levels. This candidate is then analyzed to determine the "optimal" sequence and that sequence is used to replace the corresponding fragments in the original gene. This shot-gun approach is laborious.

These tedious strategies are employed to amplify the synthesis of a desired protein which is produced by the unaltered (native) gene only in small quantities. The thermostable DNA polymerase from Thermus aquaticus (Taq Pol) is such a product.

Taq Pol catalyzes the combination of nucleotide triphosphates to form a nucleic acid strand complementary to a nucleic acid template strand. The application of thermostable Taq Pol to the amplification of nucleic acid by polymerase chain reaction (PCR) was the key step in the development of PCR to its now dominant position in molecular biology. The gene encoding Taq Pol has been cloned, sequenced, and expressed in E. coli, yielding only modest amounts of Taq Pol.

The problem is that although Taq Pol is commercially available from several sources, it is expensive, partly because of the modest amounts recovered by using the methods currently available. Increased production of Taq Pol is clearly desirable to meet increasing demand and to make production more economical.

FIG. 1, the sole illustration, shows the relevant genetic components of a vector, pSCW562, used to transform an E. coli host.

The present invention provides a gene for Taq polymerase wherein the sequence of the first thirty nucleotide bases in the native gene which code for the first ten amino acids in the mature native protein, has been changed

A) by substituting therefor a modified nucleotide sequence selected from the group consisting of:

SEQ ID NO: 2:

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG , and 33

SEQ ID NO: 4:

ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG 36

CTG CCC CTC TTT GAG CCC AAG , 57

or

B) by inserting between the codon (ATG) for the first amino acid of the mature native protein and the codon, (AGG) for the second amino acid of the mature native protein, the sequence:

5

SEQ ID NO:8 :

GAC TAC AAG GAC GAC GAT GAC AAG .

24

The invention also provides a method of increasing the production of Taq Pol by using the above altered genes.

10 The invention provides enhanced polymerase activity levels as high as 200-fold. The recombinant polymerase of this invention is functionally indistinguishable from native Taq Pol.

1. Introduction

15 The object of the present invention is to increase the production of Taq polymerase in E. coli by changing selected nucleotide sequences in the 5' region of the gene which encode the N-terminus of the polymerase.

The invention provides four nucleotide sequences which differ from the native Thermus aquaticus polymerase (Taq Pol) gene in one to

20 several nucleotides. When introduced into the native gene and transfected into E. coli, these DNA sequences provide improved expression of the gene, evidenced by increased activity of the enzyme. The amount of increase varies widely depending on the nucleotide changes made and also on other factors such as induction with IPTG, incubation period of E. coli, and so forth.

25 The genes provided by the present invention are the same as the native Taq Pol gene except for changes in the native sequence made in accordance with the present invention. Where these changes are made, they are specifically described and shown in the examples and in the Sequence Listing. Changes are only in the region encoding the N-terminus of the protein. More specifically, changes are made only in the region upstream of the eleventh codon (AAG) coding for the eleventh amino acid (lysine) in the mature native protein. The eleventh codon is not changed, but it is shown in the sequence listing as the bracket or the point above which changes are made in the practise of the invention. Except for these identified changes, the remaining sequence of the Taq Pol gene remains unchanged.

30 The term "Taq Pol gene" as used herein refers to the nucleotide sequence coding for the thermostable DNA polymerase of Thermus aquaticus and includes mutant forms, spontaneous or induced, of the native gene as long as the mutations do not confer substantial changes in the essential activity of the native polymerase

The term "Tag Pol" as used herein refers to the polymerase encoded by the Taq Pol gene.

35 The term "native" as used herein refers to the unaltered nucleotide sequence of the Taq Pol gene or the unaltered amino acid sequence of the Taq polymerase as that gene or enzyme occurs naturally in T. aquaticus. See SEQ ID NO:1. In general terms, the invention comprises the following steps:

A) providing a vector with a Taq Pol gene of the invention,

40 B) transfecting compatible E. coli host cells with the vector of A) thereby obtaining transformed E. coli host cells; and
C) culturing the transformed cells of B) under conditions for growth thereby producing Taq polymerase synthesized by the transformed host cells.

The following bacterial strains, plasmids, phage and reagents were used in the invention.

45 2. Bacterial Strains

Thermus aquaticus YT-I, ATCC No. 25104, was used for native DNA isolation. The host E. coli strain for all cloning and plasmid manipulation, DH5 α [F⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(r_K⁻, m_K⁺) supE44 thi gyrA relA1] was obtained from BRL.

50 Strain JM103 [thi⁻, strA, supE, endA, sbcB, hsdR⁻, D(lac-pro), F' traD36, proAB, lacI^q, lacZDM15) (Yanisch-Perron and others, Improved M13 Phage Cloning Vectors and Host Strains: Nucleotide Sequences of M13mp18 and pUC19 Vectors, Gene 33:103-119 (1985)) was also utilized for protein expression experiments.

The host strain for preparation of single-stranded DNA for use in mutagenesis was CJ236 (pCJ105, dut ung thi relA) (Kunkel and others, Rapid and Efficient Site-specific Mutagenesis without Phenotypic Selection, Methods Enzymol
55 154:367-382, (1987)).

The f1 phage R408 (Russel and others, An Improved Filamentous Helper Phage for Generating Single-stranded DNA, Gene 45:333-338 (1986)) was used as the helper to generate single-stranded plasmid DNA for mutagenesis. The plasmid used for all cloning and expression work was pSCW562 or its derivative pTaq1. A diagram of pSCW562 is

shown in Figure 1. When the native Taq Pol gene is inserted into pSCW562, the resulting plasmid is designated pTaq1. When the native Taq Pol gene is altered by mutagenesis, the mutant plasmid is designated pTaq3, pTaq4, pTaq5, or pTaq6 depending on the nucleotide sequence with which it is mutagenized.

3. Reagents

Chemicals were purchased from Sigma, International Biotechnologies, Inc. or Eastman Kodak. LB medium was obtained from Gibco. Enzymes were purchased from New England Biolabs, IBI, BRL, Boehringer-Mannheim, or U.S. Biochemicals and were used as recommended by the supplier. Sequenase™ kits for DNA sequencing were obtained from U.S. Biochemicals. Radioisotopes were purchased from Amersham. Taq polymerase was purchased from Cetus.

4. Method of Increasing the Production of Taq Pol

Step A - Providing a Vector with the Taq Pol Gene of the Invention

One method of providing a vector with the Taq Pol gene of the invention is to:

- provide the native DNA from Thermus aquaticus;
- amplify the native Taq Pol DNA and incorporate restriction sites at both ends of the DNA fragments,
- ligate the DNA fragments of ii) into a suitable vector,
- use site-directed mutagenesis to change the nucleotide sequence of the native DNA, and
- screen for vectors carrying the changed nucleotide sequence of the invention.

i. Providing the Native Gene from T. aquaticus

All DNA manipulations were done using standard protocols (Maniatis and others, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982 and Ausubel and others, Current Protocols in Molecular Biology, John Wiley and Sons, New York, New York, 1987). Total DNA from T. aquaticus (strain YT-1, [ATCC No. 25104]) was isolated from a 40 mL culture of the organism grown overnight at 70°C in ATCC medium #461. The cells were pelleted by centrifugation, washed once with 10 mM Tris HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris HCl (pH 8.0) (TE), and resuspended in 5 mL of TE. Lysozyme was added to a concentration of 1 mg/mL and the solution was incubated at 37°C for 30 minutes. EDTA, sodium dodecyl sulfate (SDS) and proteinase K were added to concentrations of 50 mM, 0.5% and 100 µg/mL, respectively, and the solution was incubated for 4 hours at 50°C. The sample was extracted three times with phenol-chloroform and once with chloroform and the DNA was precipitated by addition of sodium acetate to 0.3 M and two volumes of ethanol. The DNA was collected by spooling on a glass rod, washed in 70% ethanol, and dissolved in (TE).

ii. Amplifying the Native Taq Pol Gene and Incorporating Restriction Sites

The fastest approach to producing large amounts of Taq Pol gene is to utilize the published nucleic acid sequence of the gene (Lawyer and others, Isolation, Characterization and Expression in Escherichia coli of the DNA Polymerase from Thermus aquaticus, Journal of Biological Chemistry, 264:6427-6437, 1989) to design oligonucleotide primers that can be used in PCR to amplify genomic DNA. See SEQ ID NO: 1: for entire gene sequence.

PCR is an amplification technique well known in the art (Saiki and others, Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase, Science 239:487-491 (1988)), which involves a chain reaction producing large amounts of a specific known nucleic acid sequence. PCR requires that the nucleic acid sequence to be amplified must be known in sufficient detail so that oligonucleotide primers can be prepared which are sufficiently complementary to the desired nucleic acid sequences, as to hybridize with them and synthesize extension products.

Primers are oligonucleotides, natural or synthetic, which are capable of acting as points of initiation for DNA synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, that is, in the presence of four different nucleotide triphosphates and thermostable enzymes in an appropriate buffer and at a suitable temperature.

PCR amplification was carried out on the Taq Pol DNA of i) essentially as described by Saiki and others, in an Ericomp thermocycler. Primers were designed based upon the published sequence of the Taq Pol gene (Lawyer and others). Amplification mixtures contained approximately 100 ng of T. aquaticus DNA, 1 µM of each of the two primers, 200 µM each of dATP, dGTP, dCTP and dTTP, and 2 units of Taq Pol in a volume of 0.05 mL. The mixtures were heated to 97°C for 10 seconds, annealed at 40°C for thirty seconds, and extended at 72°C for 5 minutes for 5 cycles. For the subsequent 20 cycles, the annealing temperature was raised to 55°C and the extension time reduced to 3 minutes.

Finally, the mixtures were incubated at 72°C for 15 minutes to maximize the amount of fully double-stranded product. The entire PCR reaction mixture was fractionated on a 1.0% agarose gel and the 2.5 kb Taq polymerase gene was cut out and extracted. DNA fragments were isolated from agarose gels using a "freeze-squeeze technique". Agarose slices were minced, frozen on dry ice, and rapidly thawed at 37°C for five minutes. The slurry was filtered by centrifugation through a Millipore 0.45 mm Durapore membrane. The filtrate was extracted once with water saturated phenol, once with phenol-chloroform (1:1), and once with chloroform. The DNA was recovered by ethanol precipitation.

Incorporating Restriction Sites: To allow excision and recovery of the Taq Pol gene during PCR and also to afford convenient cloning of the Taq Pol gene into an expression vector, two restriction sites were introduced at the 5' ends of both strands of the gene. More specifically, one restriction site was introduced adjacent to and upstream from the start (ATG) codon and the other restriction site was introduced adjacent to and downstream from the stop (TGA) codon (SEQ ID NOS: 6 & 7). The nucleotides forming the restriction sites were included on the synthetic primer used in the PCR. In the examples disclosed herein, the nucleotide sequence GAATTC, which forms EcoR1 restriction site was included on the primers.

Other restriction sites may be used in the practice of this invention provided that 1) the expression vector has a corresponding site where the Taq DNA is to be ligated, 2) the restriction site does not occur within the Taq Pol gene.

As shown in Figure 1, EcoR1 is one of several restriction sites in pSCW562. Other exemplary restriction sites are XbaI and SphI. Of course, expression vectors having other restriction sites would provide still more potential restriction sites which would be useful in the practice of this invention.

When digested with the appropriate enzyme, these restriction sites form sticky ends which can be conveniently ligated to correspondingly digested restriction sites on the expression vector. The restriction sites do not affect the amino acid sequence of Taq Pol.

Alternative Method: In lieu of the PCR technique described above, the native Taq Pol gene may alternatively be provided by conventionally cloning the gene. In that event, the restriction sites may be introduced by site directed mutagenesis. The end results of either procedure are indistinguishable.

iii. Ligating DNA Fragments into a Vector

The DNA from step ii) is then ligated to a suitable expression vector. The vector chosen for cloning was pSCW562, which contains an EcoR1 site 11 base pairs downstream of the ribosome binding site and the strong tac (trp-lac hybrid) promoter (Figure 1). The Taq Pol gene does not contain any EcoR1 sites, so the PCR primers were designed with EcoR1 sites near their 5' ends (step ii)) to allow direct cloning into the EcoR1 site of pSCW562.

In addition to the EcoR1 site, vector pSCW562 contains 1) a phage origin of replication (F_1), 2) a plasmid origin of replication (ORI), 3) an antibiotic resistance marker (AMP), and 4) a transcription termination sequence downstream of the restriction sites. This plasmid was constructed using techniques well known in the art of recombinant DNA as taught in Maniatis and others, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York (1982). However, this particular plasmid is not critical to the invention. Any vector containing an appropriate promoter and restriction sites will be useful in this method.

The EcoR1-digested PCR product from Step ii) was fractionated in a 1% agarose gel and eluted. The vector, pSCW562, was digested overnight with EcoR1 (10 units/ μ g) and treated with calf intestinal alkaline phosphatase (1 unit/ μ g), extracted with phenol/chloroform, ethanol precipitated, and resuspended in TE. Approximately 200 ng of the prepared vector was mixed with 500 ng of purified PCR product and ligated for 18 hours in 50 mM TrisHCl, pH 7.8, 10 mM MgCl₂, 20 mM dithiothreitol, 1mM ATP, with 0.5 Weiss units of T4 DNA ligase in a volume of 20 μ L.

iv. Using Site-Directed Mutagenesis to Change the Nucleotide Sequence of the Native Taq Pol Gene

Site-directed mutagenesis is a method of altering the nucleotide sequence of a DNA fragment by specifically substituting, inserting or deleting selected nucleotides within the sequence to be altered. The method involves priming in vitro DNA synthesis with chemically synthesized nucleotides that carry a nucleotide mismatch with the template sequence. The synthetic oligonucleotide primes DNA synthesis and is itself incorporated into the resulting heteroduplex molecule. After transformation of host cells, this heteroduplex gives rise to homoduplexes whose sequences carry the mutagenic nucleotides. Mutant clones are selected by screening procedures well known in the art such as nucleic acid hybridization with labelled probes and DNA sequencing.

Using site-directed mutagenesis, we constructed mutant genes for Taq polymerase wherein the sequence of the first thirty nucleotide bases in the native gene which code for the first ten amino acids in the mature native protein, was changed

A) by substituting therefor a modified nucleotide sequence selected from the group consisting of:

Example 1 - SEQ ID NO: 2:

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG , 33

Example 2 - SEQ ID NO: 4:

ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG 36

CTG CCC CTC TTT GAG CCC AAG , 57

or, Example 3,

B) by inserting between the start codon (ATG) for the first amino acid of the mature native protein and the codon, (AGG) for the second amino acid of the mature native protein, the sequence:

SEQ ID NO: 8 :

GAC TAC AAG GAC GAC GAT GAC AAG . 24

In the examples above, bases that are changed are highlighted in bold type. The effect that these changes have on polymerase activity is shown in Table I. The above examples are offered by way of illustration only and are by no means intended to limit the scope of the claimed invention.

In these examples all gene modifications were carried out by site-directed mutagenesis. However, alternative methods are known in the art which would give the same results. For example, the changes to the Taq Pol gene described above could have been incorporated directly into the gene during amplification (PCR) by appropriately designing the upstream oligonucleotide primer to include the nucleotide sequences of the invention.

Another alternative would be to incorporate unique restriction sites bracketing the first ten codons of the gene. This would allow removal of the sequences encoding the amino terminus by restriction endonuclease cleavage and replacement using a double stranded synthetic fragment. Either of these methods could be used to accomplish the nucleotide changes set forth above.

Site-directed mutagenesis was carried out essentially as described by Kunkel and others, Rapid and Efficient Site-specific Mutagenesis without Phenotypic Selection, Methods Enzymol, 154:367-382, (1987), using a kit obtained from Bio Rad. Single-stranded plasmid DNA was prepared by infecting early exponential phase cultures of CJ236 (carrying pTaq1) with R408 at a multiplicity of infection of approximately 10-20. After overnight growth at 37°C, the cells were removed by centrifugation and the phage precipitated by addition of polyethylene glycol to 5% and NaCl to 0.5 M. The phage were pelleted by centrifugation and the DNA isolated by phenol-chloroform extraction and ethanol precipitation. The mutagenic oligonucleotides were phosphorylated with T4 polynucleotide kinase and 9 pmol of each was annealed to approximately 3 pmol of single-stranded plasmid DNA. The annealed mixture was extended with T4 DNA polymerase, ligated, and transformed into DH5α or JM103. Plasmid DNA was isolated from the transformants by rapid boiling (Holmes and Quigley, A Rapid Boiling Method for the Preparation of Bacterial Plasmids, Anal. Biochem. 114:193-199, 1981) and digested with EcoR1 to identify clones that had undergone mutagenesis.

v. Screening for Vectors with the Taq Pol Gene

To verify that the clones of iv) were carrying the desired Taq Pol gene, clones were lifted on to nitrocellulose filters and identified as Taq Pol transformants by colony hybridization.

Colony Hybridization: This technique identifies a specific nucleic acid sequence by creating conditions for single strands of the specific nucleic acid sequence to base pair (hybridize) with a complementary radioactive single stranded nucleic acid fragments (probes). Double-stranded regions form where the two types of DNA have complementary nucleotide sequences and are detected by their radioactivity.

Colonies containing the Taq Pol fragment were identified by hybridization with an internal oligonucleotide:

SEQ ID NO: 10:

GTGGTCTTTG ACGCCAAG,

labelled with ³²P at the 5' end with T4 polynucleotide kinase. Colony hybridizations were performed as described in

Maniatis and others, *supra* in 5X SSPE [1XSSPE in 10 mM sodium phosphate, pH 7.0, 0.18 M NaCl, 1 mM EDTA], 0.1% sodium lauroyl sarcosine, 0.02% SDS, 0.5% blocking agent (Boehringer-Mannheim) containing approximately 5 ng per mL ^{32}P labelled oligonucleotide. Hybridization was conducted at 42°C for 4-18 hours. The filters were washed in 2X SSPE, 0.1% SDS at room temperature three times, followed by a stringent wash at 42°C in the same solution.

Positive colonies were identified by autoradiography.

Sequence Analysis: To ascertain whether or not the Taq Pol DNA was incorporated in the correct orientation, DNA sequence analysis was performed on alkaline denatured supercoiled DNA as described by Zhang and others, Double Stranded DNA sequencing as a Choice for DNA Sequencing, Nucleic Acids Research 16:1220 (1988), using a Sequenase™ kit from U.S. Biochemicals and a (^{35}S)dATP. Typically, 1.0 μL of supercoiled, CsCl-banded DNA was denatured in 20 μL of 0.2 M NaOH, 0.2 mM EDTA for 5 minutes. The solution was neutralized with 2 μL of 2 M ammonium acetate (pH 4.6) and precipitated with 60 mL of ethanol. The mixture was centrifuged for 10 minutes, washed once with 80% ethanol, dried for 10 minutes and resuspended in 7 mL of H_2O . After addition of 5 ng of primer and 2 μL of 5X buffer, the samples were heated to 65°C and allowed to cool to < 37°C over 30-45 minutes. The sequencing reactions were then performed as directed by the supplier. The reactions were then performed as directed by the supplier. The reactions were electrophoresed on 6% sequencing gels, occasionally utilizing a sodium acetate salt gradient to improve resolution near the bottom of the gel (Sheen and others, Electrolyte Gradient Gels for DNA Sequencing, Bio Techniques 6:942-944, 1989). Alternatively, plasmid DNA prepared by the rapid boiling or alkaline miniprep procedures was used for sequencing after extraction with phenol-chloroform and ethanol precipitation, although with some reduced reliability.

Step B - Transfecting Host Cells with the Vector of A)

The vector of step A) is used to transfect a suitable host and the transformed host is cultured under favorable conditions for growth. Prokaryotic hosts are in general the most efficient and convenient in genetic engineering techniques and are therefore preferred for the expression of Taq polymerase. Prokaryotes most frequently are represented by various strains of *E. coli* such as DH5a and JM103, the strains used in the examples below. However, other microbial strains may also be used, as long as the strain selected as host is compatible with the plasmid vector with which it is transformed. Compatibility of host and plasmid/vector means that the host faithfully replicates the plasmid/vector DNA and allows proper functioning of the above controlling elements. In our system, DH5 α and JM103 are compatible with pSCW562.

Five mL of the ligation mixture of Step B were mixed with 0.1 μL of DH5 α or JM103 cells made competent by CaCl_2 treatment as described by Cohen and others, Proc. National Academy of Science, USA, 69:2110 (1972). After incubation on ice for 15-30 minutes, the mixture was incubated at 42°C for 90 seconds. After the heat shock, one mL of LB medium was added and the cells were incubated for one hour at 37°C.

Selection of Transformants: After the one-hour incubation, aliquots of the incubated mixture were spread on LB agar plates containing 50 $\mu\text{g}/\text{mL}$ ampicillin and incubated at 37°C for 18 hours. Only transformed *E. coli* carrying the AMP (marker) gene can grow on this medium. To select transformants that were also carrying the Taq Pol gene in correct orientation, colony hybridization and sequence analysis were done using techniques already described above.

Step C - Culturing the Transformed Hosts

E. coli transformants verified as containing the Taq Pol gene in the correct orientation, were cultured in 40 mL of LB broth at 37°C to mid-log phase and where appropriate, were induced with 1 mM isopropyl- β -D-thiogalactoside (IPTG). The cells were allowed to grow for either an additional two hours or overnight, and were harvested by centrifugation. The cells were resuspended in 0.25 mL of 50 mM TrisHCl, pH 7.5, 1 mM EDTA, 0.5 $\mu\text{g}/\text{mL}$ leupeptin, 2.4 mM phenylmethylsulphonyl fluoride and sonicated. The lysate was diluted with 0.25 mL of 10 mM TrisHCl, pH 8.0, 50 mM KCl, 0.5% Tween 20, 0.5% NP-40 and heated to 74°C for 20 minutes. After cooling on ice for 15 minutes, the debris was removed by centrifugation for 10 minutes at 4°C. Aliquots of the supernatant fraction were assayed for DNA polymerase activity using activated salmon sperm DNA as the substrate.

DNA Polymerase Assay: This assay is based on the ability of DNA polymerases to fill in single strand gaps made in double stranded DNA. It uses the single strand gaps as templates and the free 3' hydroxyl group at the border of the single strand gap as the primer at which it begins synthesis. Specifically, 5 μL of enzyme preparation was incubated for 10 minutes at 74°C in a total of 50 μL with the following: 25 mM Tris(hydroxymethyl)methyl-3-amino-propane sulfonic acid (TAPS) (pH 9.8 at 22°C), 50 mM KCl, 1 mM 2-mercaptoethanol, 2 mM MgCl_2 0.30 mg/mL activated salmon testes DNA, 0.2 mM of each dCTP, dGTP, dTTP, and 0.1 mM (200 nCi/nmol) [^3H]dATP. The reaction was stopped by the addition of 100 μL of 0.15 M sodium pyrophosphate, 0.105 M sodium EDTA, pH 8.0, followed by the addition of ice cold 10% trichloroacetic acid (TCA). It was then kept on ice for 15-30 minutes prior to being vacuum filtered on a prewet 25 mm Whatman glass fiber filters (GFC) filter disk. The precipitated reaction product was washed free of unincorporated ^3H on the filter with a total of 12 mL of ice cold 10% TCA followed by a total of 12 mL of ice cold 95% ethanol. Filters were

vacuum dried, then air dried, and then counted directly in a scintillation fluid. Enzyme preparations that required diluting were diluted with a solution of 10 mM Tris, 50 mM KCl, 10 mM MgCl₂, 1.0 mg/mL gelatin, 0.5% nonidet P40, 0.5% Tween 20, 1 mM 2-mercaptoethanol, pH 8.0. One unit of activity is the amount of enzyme required to incorporate 10 nmol of total nucleotide in 30 min at 74°C; adenine constitutes approximately 29.7% of the total bases in salmon sperm DNA.

Salmon testes DNA (Sigma type III; product #D1626) was dissolved to 1.3 mg/mL in TM buffer (10 mM Tris, 5 mM MgCl₂, pH 7.2) and stirred slowly for 24 hours at 4°C. It was then diluted 2.5 fold with TM buffer and made 0.3 M in NaCl prior to extracting at room temperature with an equal volume of phenol/chloroform (1:1::vol:vol; phenol saturated with TM buffer). The mixture was centrifuged at 2700 x g for 5 minutes at room temperature to aid separation of the phases, the aqueous phase was collected and extracted with an equal volume of chloroform. The mixture was centrifuged as above and the aqueous phase again collected. The activated DNA in the aqueous phase was precipitated with two volumes of 95% ethanol at -20°C; the precipitated mixture was kept at -20°C for 12-18 hours. The precipitated DNA was collected by centrifuging at 13,700 x g for 30 minutes at 2°C. The pellet was dried with a stream of nitrogen gas and then redissolved 3-6 mg/mL with TE (10 mM Tris, 1 mM EDTA, pH 7.5) with slow rocking for 12-18 hours at room temperature. The solution was dialyzed against TE and then adjusted to the proper concentration by checking the absorbance at 260 nm. Aliquots (0.5-1.0 mL) were stored at -20°C; for use, one vial was thawed and then kept at 4°C rather than refreezing.

5. Results of Polymerase Assay

The results of the Taq Pol assay are shown in Table I. Vector pTaq1 carries SEQ ID NO:1 which is the native Taq Pol sequence, while the other four plasmids carry sequences which are altered in accordance with the invention as described above.

Table I shows, unexpectedly, that pTaq3 (SEQ ID NO: 2) expressed Taq Pol activity up to 200 times that of pTaq1; pTaq4 (SEQ ID NO: 3) had about 10 times the activity of pTaq1; pTaq5 (SEQ ID NO: 4) was about 10 - 50 times greater than pTaq1, depending on the experiment, and pTaq6 (SEQ NO: 5) was at least 10 times as great as pTaq1 (SEQ ID NO: 1). These results are unexpected.

The short nucleotide sequences in the Sequence Listing represent sequence changes in the first 30 nucleotides of the native gene. It is to be understood that these sequences represent only a small fraction of the complete Taq Pol gene which in its entirety contains over 2,000 nucleotides.

TABLE I

(Units/mg of protein)							
Host Strain:							
Time of Harvest: Induction Plasmid	DH5α O/N -	DH5α O/N +	JM103 2 Hrs. +	JM103 2 Hrs. +	JM103 O/N +	JM103 2 Hrs. -	JM103 2 Hrs. +
<u>SEQ ID NO: 1</u> pTaq1	40	90	100	270	1030	60	180
<u>SEQ ID NO: 2</u> pTaq3	7290	19240	4150	4510	27420	11400	21810
<u>SEQ ID NO: 3</u> pTaq4	470	1050	1080	1570	5080	900	2360
<u>SEQ ID NO: 4</u> pTaq5	ND	ND	6060	4610	14190	3500	10700
<u>SEQ ID NO: 5</u> pTaq6	2486	7644	ND	ND	ND	ND	ND

ND = not determined

ON = overnight

+ = induction

- = no induction

Table I - Assay of thermostable DNA polymerase activity encoded by the various expression plasmids. Polymerase activity is interpreted as a reflection of gene expression and polymerase production.

SEQUENCE IDENTIFICATION

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Sullivan, Mark Alan
(ii) TITLE OF INVENTION: Increased Production of Thermus aquaticus DNA Polymerase in E. coli.
(iii) NUMBER OF SEQUENCES: 14
(iv) CORRESPONDENCE ADDRESS:

- 10 (A) ADDRESSEE: Eastman Kodak Company, Patent Department
(B) STREET: 343 State Street
(C) CITY: Rochester
(D) STATE: New York
(E) COUNTRY: U.S.A.
15 (F) ZIP: 14650-2201

(v) COMPUTER READABLE FORM:

- 20 (A) MEDIUM TYPE: Diskette, 3.5 inch, 800 Kb storage
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 6.0
(D) SOFTWARE: WriteNow

(vi) CURRENT APPLICATION DATA:

- 25 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

- 30 (vii) PRIOR APPLICATION DATA: None
(viii) ATTORNEY/AGENT INFORMATION

- (A) NAME: Wells, Doreen M.
(B) REGISTRATION NUMBER: 34,278
35 (C) REFERENCE/DOCKET NUMBER: 58374D-W1100

(ix) TELECOMMUNICATION INFORMATION:

- 40 (A) TELEPHONE: (716) 477-0554
(B) TELEFAX: (716) 477-4646

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS

- 45 (A) LENGTH: 2499
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

- 50 (ii) MOLECULE TYPE: genomic DNA
(iii) HYPOTHETICAL: no
(iv) ANTI-SENSE: no
(vi) ORIGINAL SOURCE:

- 55 (A) ORGANISM: *Thermus aquaticus*
(B) ISOLATE: YT1, ATCC 25104

EP 0 482 714 B1

(vii) IMMEDIATE SOURCE: amplified from genomic DNA

(ix) FEATURE:

(A) NAME/KEY: peptide

(B) LOCATION: 1-2496

(C) IDENTIFICATION METHOD: comparison to sequence in GenBank, Accession number J04639.

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K., Drummond, R., Gelfand, D.H.

(B) TITLE: Isolation, characterization and expression in Escherichia coli of the DNA polymerase gene from Thermus aquaticus.

(C) JOURNAL: Journal of Biological Chemistry

(D) VOLUME: 264

(E) ISSUE: 11

(F) PAGES: 6427-6437

(G) DATE: 15-Apr-1989

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1 :

	ATG	AGG	GGG	ATG	CTG	CCC	CTC	TTT	GAG	CCC	AAG	GGC	CGG	GTC	CTC	45
	Met	Arg	Gly	Met	Leu	Pro	Leu	Phe	Glu	Pro	Lys	Gly	Arg	Val	Leu	
	1				5					10					15	
5	CTG	GTG	GAC	GGC	CAC	CAC	CTG	GCC	TAC	CGC	ACC	TTC	CAC	GCC	CTG	90
	Leu	Val	Asp	Gly	His	His	Leu	Ala	Tyr	Arg	Thr	Phe	His	Ala	Leu	
					20					25					30	
10	AAG	GGC	CTC	ACC	ACC	AGC	CGG	GGG	GAG	CCG	GTG	CAG	GCG	GTC	TAC	135
	Lys	Gly	Leu	Thr	Thr	Ser	Arg	Gly	Glu	Pro	Val	Gln	Ala	Val	Tyr	
					35					40					45	
15	GGC	TTC	GCC	AAG	AGC	CTC	CTC	AAG	GCC	CTC	AAG	GAG	GAC	GGG	GAC	180
	Gly	Phe	Ala	Lys	Ser	Leu	Leu	Lys	Ala	Leu	Lys	Glu	Asp	Gly	Asp	
					50					55					60	
20	GCG	GTG	ATC	GTG	GTC	TTT	GAC	GCC	AAG	GCC	CCC	TCC	TTC	CGC	CAC	225
	Ala	Val	Ile	Val	Val	Phe	Asp	Ala	Lys	Ala	Pro	Ser	Phe	Arg	His	
					65					70					75	
25	GAG	GCC	TAC	GGG	GGG	TAC	AAG	GCG	GGC	CGG	GCC	CCC	ACG	CCG	GAG	270
	Glu	Ala	Tyr	Gly	Gly	Tyr	Lys	Ala	Gly	Arg	Ala	Pro	Thr	Pro	Glu	
					80					85					90	
30	GAC	TTT	CCC	CGG	CAA	CTC	GCC	CTC	ATC	AAG	GAG	CTG	GTG	GAC	CTC	315
	Asp	Phe	Pro	Arg	Gln	Leu	Ala	Leu	Ile	Lys	Glu	Leu	Val	Asp	Leu	
					95					100					105	
35	CTG	GGG	CTG	GCG	CGC	CTC	GAG	GTC	CCG	GGC	TAC	GAG	GCG	GAC	GAC	360
	Leu	Gly	Leu	Ala	Arg	Leu	Glu	Val	Pro	Gly	Tyr	Glu	Ala	Asp	Asp	
					110					115					120	
40	GTC	CTG	GCC	AGC	CTG	GCC	AAG	AAG	GCG	GAA	AAG	GAG	GGC	TAC	GAG	405
	Val	Leu	Ala	Ser	Leu	Ala	Lys	Lys	Ala	Glu	Lys	Glu	Gly	Tyr	Glu	
					125					130					135	
45	GTC	CGC	ATC	CTC	ACC	GCC	GAC	AAA	GAC	CTT	TAC	CAG	CTC	CTT	TCC	450
	Val	Arg	Ile	Leu	Thr	Ala	Asp	Lys	Asp	Leu	Tyr	Gln	Leu	Leu	Ser	
					140					145					150	
50	GAC	CGC	ATC	CAC	GTC	CTC	CAC	CCC	GAG	GGG	TAC	CTC	ATC	ACC	CCG	495
	Asp	Arg	Ile	His	Val	Leu	His	Pro	Glu	Gly	Tyr	Leu	Ile	Thr	Pro	
					155					160					165	
55																

	GCC	TGG	CTT	TGG	GAA	AAG	TAC	GGC	CTG	AGG	CCC	GAC	CAG	TGG	GCC	540
	Ala	Trp	Leu	Trp	Glu	Lys	Tyr	Gly	Leu	Arg	Pro	Asp	Gln	Trp	Ala	
					170					175					180	
5	GAC	TAC	CGG	GCC	CTG	ACC	GGG	GAC	GAG	TCC	GAC	AAC	CTT	CCC	GGG	585
	Asp	Tyr	Arg	Ala	Leu	Thr	Gly	Asp	Glu	Ser	Asp	Asn	Leu	Pro	Gly	
					185					190					195	
10	GTC	AAG	GGC	ATC	GGG	GAG	AAG	ACG	GCG	AGG	AAG	CTT	CTG	GAG	GAG	630
	Val	Lys	Gly	Ile	Gly	Glu	Lys	Thr	Ala	Arg	Lys	Leu	Leu	Glu	Glu	
					200					205					210	
15	TGG	GGG	AGC	CTG	GAA	GCC	CTC	CTC	AAG	AAC	CTG	GAC	CGG	CTG	AAG	675
	Trp	Gly	Ser	Leu	Glu	Ala	Leu	Leu	Lys	Asn	Leu	Asp	Arg	Leu	Lys	
					215					220					225	
20	CCC	GCC	ATC	CGG	GAG	AAG	ATC	CTG	GCC	CAC	ATG	GAC	GAT	CTG	AAG	720
	Pro	Ala	Ile	Arg	Glu	Lys	Ile	Leu	Ala	His	Met	Asp	Asp	Leu	Lys	
					230					235					240	
	CTC	TCC	TGG	GAC	CTG	GCC	AAG	GTG	CGC	ACC	GAC	CTG	CCC	CTG	GAG	765
	Leu	Ser	Trp	Asp	Leu	Ala	Lys	Val	Arg	Thr	Asp	Leu	Pro	Leu	Glu	
					245					250					255	
25	GTG	GAC	TTC	GCC	AAA	AGG	CGG	GAG	CCC	GAC	CGG	GAG	GGG	CTT	AGG	810
	Val	Asp	Phe	Ala	Lys	Arg	Arg	Glu	Pro	Asp	Arg	Glu	Gly	Leu	Arg	
					260					265					270	
30	GCC	TTT	CTG	GAG	AGG	CTT	GAG	TTT	GGC	AGC	CTC	CTC	CAC	GAG	TTC	855
	Ala	Phe	Leu	Glu	Arg	Leu	Glu	Phe	Gly	Ser	Leu	Leu	His	Glu	Phe	
					275					280					285	
35	GGC	CTT	CTG	GAA	AGC	CCC	AAG	GCC	CTG	GAG	GAG	GCC	CCC	TGG	CCC	900
	Gly	Leu	Leu	Glu	Ser	Pro	Lys	Ala	Leu	Glu	Glu	Ala	Pro	Trp	Pro	
					290					295					300	
40	CCG	CCG	GAA	GGG	GCC	TTC	GTG	GGC	TTT	GTG	CTT	TCC	CGC	AAG	GAG	945
	Pro	Pro	Glu	Gly	Ala	Phe	Val	Gly	Phe	Val	Leu	Ser	Arg	Lys	Glu	
					305					310					315	
	CCC	ATG	TGG	GCC	GAT	CTC	CTC	GCC	CTG	GCC	GCC	GCC	AGG	GGG	GGC	990
	Pro	Met	Trp	Ala	Asp	Leu	Leu	Ala	Leu	Ala	Ala	Ala	Arg	Gly	Gly	
					320					325					330	
45	CGG	GTC	CAC	CGG	GCC	CCC	GAG	CCT	TAT	AAA	GCC	CTC	AGG	GAC	CTG	1035
	Arg	Val	His	Arg	Ala	Pro	Glu	Pro	Tyr	Lys	Ala	Leu	Arg	Asp	Leu	
					335					340					345	
50	AAG	GAG	GCG	CGG	GGG	CTT	CTC	GCC	AAA	GAC	CTG	AGC	GTT	CTG	GCC	1080
	Lys	Glu	Ala	Arg	Gly	Leu	Leu	Ala	Lys	Asp	Leu	Ser	Val	Leu	Ala	
					350					355					360	

55

	CTG	AGG	GAA	GGC	CTT	GGC	CTC	CCG	CCC	GGC	GAC	GAC	CCC	ATG	CTC	1125
	Leu	Arg	Glu	Gly	Leu	Gly	Leu	Pro	Pro	Gly	Asp	Asp	Pro	Met	Leu	
				365						370					375	
5	CTC	GCC	TAC	CTC	CTG	GAC	CCT	TCC	AAC	ACC	ACC	CCC	GAG	GGG	GTG	1170
	Leu	Ala	Tyr	Leu	Leu	Asp	Pro	Ser	Asn	Thr	Thr	Pro	Glu	Gly	Val	
				380						385					390	
10	GCC	CGG	CGC	TAC	GGC	GGG	GAG	TGG	ACG	GAG	GAG	GCG	GGG	GAG	CGG	1215
	Ala	Arg	Arg	Tyr	Gly	Gly	Glu	Trp	Thr	Glu	Glu	Ala	Gly	Glu	Arg	
					395					400					405	
15	GCC	GCC	CTT	TCC	GAG	AGG	CTC	TTC	GCC	AAC	CTG	TGG	GGG	AGG	CTT	1260
	Ala	Ala	Leu	Ser	Glu	Arg	Leu	Phe	Ala	Asn	Leu	Trp	Gly	Arg	Leu	
					410					415					420	
	GAG	GGG	GAG	GAG	AGG	CTC	CTT	TGG	CTT	TAC	CGG	GAG	GTG	GAG	AGG	1305
	Glu	Gly	Glu	Glu	Arg	Leu	Leu	Trp	Leu	Tyr	Arg	Glu	Val	Glu	Arg	
					425					430					435	
20	CCC	CTT	TCC	GCT	GTC	CTG	GCC	CAC	ATG	GAG	GCC	ACG	GGG	GTG	CGC	1350
	Pro	Leu	Ser	Ala	Val	Leu	Ala	His	Met	Glu	Ala	Thr	Gly	Val	Arg	
					440					445					450	
25	CTG	GAC	GTG	GCC	TAT	CTC	AGG	GCC	TTG	TCC	CTG	GAG	GTG	GCC	GAG	1395
	Leu	Asp	Val	Ala	Tyr	Leu	Arg	Ala	Leu	Ser	Leu	Glu	Val	Ala	Glu	
					455					460					465	
30	GAG	ATC	GCC	CGC	CTC	GAG	GCC	GAG	GTC	TTC	CGC	CTG	GCC	GGC	CAC	1440
	Glu	Ile	Ala	Arg	Leu	Glu	Ala	Glu	Val	Phe	Arg	Leu	Ala	Gly	His	
					470					475					480	
35	CCC	TTC	AAC	CTC	AAC	TCC	CGG	GAC	CAG	CTG	GAA	AGG	GTC	CTC	TTT	1485
	Pro	Phe	Asn	Leu	Asn	Ser	Arg	Asp	Gln	Leu	Glu	Arg	Val	Leu	Phe	
					485					490					495	
	GAC	GAG	CTA	GGG	CTT	CCC	GCC	ATC	GGC	AAG	ACG	GAG	AAG	ACC	GGC	1530
	Asp	Glu	Leu	Gly	Leu	Pro	Ala	Ile	Gly	Lys	Thr	Glu	Lys	Thr	Gly	
					500					505					510	
40	AAG	CGC	TCC	ACC	AGC	GCC	GCC	GTC	CTG	GAG	GCC	CTC	CGC	GAG	GCC	1575
	Lys	Arg	Ser	Thr	Ser	Ala	Ala	Val	Leu	Glu	Ala	Leu	Arg	Glu	Ala	
					515					520					525	
45	CAC	CCC	ATC	GTG	GAG	AAG	ATC	CTG	CAG	TAC	CGG	GAG	CTC	ACC	AAG	1620
	His	Pro	Ile	Val	Glu	Lys	Ile	Leu	Gln	Tyr	Arg	Glu	Leu	Thr	Lys	
					530					535					540	
50	CTG	AAG	AGC	ACC	TAC	ATT	GAC	CCC	TTG	CCG	GAC	CTC	ATC	CAC	CCC	1665
	Leu	Lys	Ser	Thr	Tyr	Ile	Asp	Pro	Leu	Pro	Asp	Leu	Ile	His	Pro	
					545					550					555	
55																

	AGG	ACG	GGC	CGC	CTC	CAC	ACC	CGC	TTC	AAC	CAG	ACG	GCC	ACG	GCC	1710
	Arg	Thr	Gly	Arg	Leu	His	Thr	Arg	Phe	Asn	Gln	Thr	Ala	Thr	Ala	
					560					565					570	
5	ACG	GGC	AGG	CTA	AGT	AGC	TCC	GAT	CCC	AAC	CTC	CAG	AAC	ATC	CCC	1755
	Thr	Gly	Arg	Leu	Ser	Ser	Ser	Asp	Pro	Asn	Leu	Gln	Asn	Ile	Pro	
					575					580					585	
10	GTC	CGC	ACC	CCG	CTT	GGG	CAG	AGG	ATC	CGC	CGG	GCC	TTC	ATC	GCC	1800
	Val	Arg	Thr	Pro	Leu	Gly	Gln	Arg	Ile	Arg	Arg	Ala	Phe	Ile	Ala	
					590					595					600	
15	GAG	GAG	GGG	TGG	CTA	TTG	GTG	GCC	CTG	GAC	TAT	AGC	CAG	ATA	GAG	1845
	Glu	Glu	Gly	Trp	Leu	Leu	Val	Ala	Leu	Asp	Tyr	Ser	Gln	Ile	Glu	
					605					610					615	
	CTC	AGG	GTG	CTG	GCC	CAC	CTC	TCC	GGC	GAC	GAG	AAC	CTG	ATC	CGG	1890
	Leu	Arg	Val	Leu	Ala	His	Leu	Ser	Gly	Asp	Glu	Asn	Leu	Ile	Arg	
					620					625					630	
20	GTC	TTC	CAG	GAG	GGG	CGG	GAC	ATC	CAC	ACG	GAG	ACC	GCC	AGC	TGG	1935
	Val	Phe	Gln	Glu	Gly	Arg	Asp	Ile	His	Thr	Glu	Thr	Ala	Ser	Trp	
					635					640					645	
25	ATG	TTC	GGC	GTC	CCC	CGG	GAG	GCC	GTG	GAC	CCC	CTG	ATG	CGC	CGG	1980
	Met	Phe	Gly	Val	Pro	Arg	Glu	Ala	Val	Asp	Pro	Leu	Met	Arg	Arg	
					650					655					660	
30	GCG	GCC	AAG	ACC	ATC	AAC	TTC	GGG	GTC	CTC	TAC	GGC	ATG	TCG	GCC	2025
	Ala	Ala	Lys	Thr	Ile	Asn	Phe	Gly	Val	Leu	Tyr	Gly	Met	Ser	Ala	
					665					670					675	
	CAC	CGC	CTC	TCC	CAG	GAG	CTA	GCC	ATC	CCT	TAC	GAG	GAG	GCC	CAG	2070
	His	Arg	Leu	Ser	Gln	Glu	Leu	Ala	Ile	Pro	Tyr	Glu	Glu	Ala	Gln	
					680					685					690	
35	GCC	TTC	ATT	GAG	CGC	TAC	TTT	CAG	AGC	TTC	CCC	AAG	GTG	CGG	GCC	2115
	Ala	Phe	Ile	Glu	Arg	Tyr	Phe	Gln	Ser	Phe	Pro	Lys	Val	Arg	Ala	
					695					700					705	
40	TGG	ATT	GAG	AAG	ACC	CTG	GAG	GAG	GGC	AGG	AGG	CGG	GGG	TAC	GTG	2160
	Trp	Ile	Glu	Lys	Thr	Leu	Glu	Glu	Gly	Arg	Arg	Arg	Gly	Tyr	Val	
					710					715					720	
45	GAG	ACC	CTC	TTC	GGC	CGC	CGC	CGC	TAC	GTG	CCA	GAC	CTA	GAG	GCC	2205
	Glu	Thr	Leu	Phe	Gly	Arg	Arg	Arg	Tyr	Val	Pro	Asp	Leu	Glu	Ala	
					725					730					735	
50	CGG	GTG	AAG	AGC	GTG	CGG	GAG	GCG	GCC	GAG	CGC	ATG	GCC	TTC	AAC	2250
	Arg	Val	Lys	Ser	Val	Arg	Glu	Ala	Ala	Glu	Arg	Met	Ala	Phe	Asn	
					740					745					750	

[illegible]

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2 :

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG 33
Met Arg Gly Met Leu Pro Leu Phe Glu Pro Lys
1 5 10

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATG CGT GGG ATG CTG CCC CTC TTT GAG CCC AAG 33
Met Arg Gly Met Leu Pro Leu Phe Glu Pro Lys
1 5 10

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 57
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10	ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG Met Asp Tyr Lys Asp Asp Asp Lys Arg Gly Met 1 5 10	36
	CTG CCC CTC TTT GAG CCC AAG Leu Pro Leu Phe Glu Pro Lys 15	57

15

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS

20

- (A) LENGTH: 57
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

30	ATG GAC TAC AAG GAC GAC GAT GAC AAG Met Asp Tyr Lys Asp Asp Asp Asp Lys 1 5	27
	AGG GGG ATG CTG CCC CTC TTT GAG CCC AAG Arg Gly Met Leu Pro Leu Phe Glu Pro Lys 10 15	57

35

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS

40

- (A) LENGTH: 20
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

45

45	GAATTC ATG AGG GGG ATG CT	20
----	----------------------------------	----

(8) INFORMATION FOR SEQ ID NO:7:

50

(i) SEQUENCE CHARACTERISTICS

55

- (A) LENGTH: 23
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGTGGGAAT TCA CTC CTT GGC GGA

23

(9) INFORMATION FOR SEQ ID NO:8:

5

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 24
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

15

GAC TAC AAG GAC GAC GAT GAC AAG
 Asp Tyr Lys Asp Asp Asp Asp Lys
 1 5

24

(10) INFORMATION FOR SEQ ID NO:9:

20

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 8 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

30

Met Asp Tyr Lys Asp Asp Asp Asp Lys
 1 5

(11) INFORMATION FOR SEQ ID NO:10:

35

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 18
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTGGTCTTTG ACGCCAAG

45

18

(12) INFORMATION FOR SEQ ID NO:11:

50

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 59
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

**AGGGGCAGCA TACCACGCTT GTCATCGTCG TCCTTGTAGT CCATAATTCT
GTTTCCTGT**

**50
59**

5

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS

10

(A) LENGTH: 59
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

**AGGGGCAGCA TCCCCCTCTT GTCATCGTCG TCCTTGTAGT CCATGAATTC
TGTTTCCTGT**

**50
60**

20

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS

25

(A) LENGTH: 48
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCCTTCGGC TCAAACAGTG GCAGCATACC ACGCATAATT CTGTTTCC

48

(15) INFORMATION FOR SEQ ID NO:14:

35

(i) SEQUENCE CHARACTERISTICS

40

(A) LENGTH: 53
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

45

**CGGCCCTTG GCTCAAAGAG GGGCAGCATC CCACGCATGA ATTCCTGTTT
CCT**

**50
53**

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Claims

1. A gene for Taq polymerase wherein the sequence of the first thirty nucleotide bases in the native gene which code for the first ten amino acids in the mature native protein, has been changed by inserting between the start codon (ATG) of the mature native protein and the codon (AGG) for the second amino acid of the mature native protein, the sequence:

55

SEQ ID NO: 8

GAC TAC AAG GAC GAC GAT GAC AAG

24.

5

2. A gene for Taq polymerase wherein the sequence of the first thirty nucleotide bases in the native gene which code for the first ten amino acids in the mature native protein, has been changed by substituting therefore the modified nucleotide sequence:

10

SEQ ID NO: 4

ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG

36

CTG CCC CTC TTT GAG CCC AAG,

57.

15

3. A gene for Taq polymerase wherein the sequence of the first thirty nucleotide bases in the native gene which code for the first ten amino acids in the mature native protein, has been changed by substituting therefore the modified nucleotide sequence:

20

SEQ ID NO: 2

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG

33.

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4. The gene of any one of claims 1 to 3, having a restriction site adjacent to and upstream from the start (ATG) codon, and the same restriction site adjacent to and downstream from the stop (TGA) codon.

5. The gene of claim 4 wherein the restriction sites are encoded by the nucleotide sequence GAATTC.

30

6. The gene of claim 3, wherein the native sequence:

SEQ ID NO: 2

ATG CGT GGT ATC CTG CCT CTG TTT GAG CCG AAG

33.

35

7. A thermostable Thermus aquaticus DNA polymerase encoded by the gene of claim 1 or claim 2, having as the first amino acid sequence in the mature protein:

SEQ ID NO: 9

Met-Asp-Tyr-Lys-Asp-Asp-Asp-Lys.

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1

5

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8. A method of increasing the production of Taq polymerase comprising the steps of:

A) providing a vector with the gene of any one of claims 1 to 3;

B) transfecting a compatible E. coli host with the vector of A) thereby obtaining transformed E. coli host cells; and

C) culturing the transformed cells of B) under conditions for growth thereby producing Taq polymerase synthesized by the transformed host cells.

50

9. The method of claim 8 wherein the vector of step A has an inducible promoter.

10. The method of claims 8 or claim 9 wherein the production of Taq polymerase is induced with isopropyl-β-D-thiogalactosidase (IPTG).

55

11. A vector with the gene of any one of claims 1 to 3, said vector having:

i) selectable markers,

- ii) a suitable promoter, and
- iii) proper regulator sequences for controlling gene expression.

12. An E. coli host cell comprising the vector of claim 11.

Revendications

1. Gène d'une Taq polymérase dans lequel la séquence des trente premières bases nucléotidiques du gène natif qui code pour les dix premiers acides aminés de la protéine mature native, a été changée en insérant entre le codon d'initiation (ATG) de la protéine mature native et le codon (AGG) du second acide aminé de la protéine mature native, la séquence :

SEQ ID n° : 8

GAC TAC AAG GAC GAC GAT GAC AAG 24 .

2. Gène d'une Taq polymérase dans lequel la séquence des trente premières bases nucléotidiques du gène natif qui code pour les dix premiers acides aminés de la protéine mature native, a été changée en substituant par conséquent la séquence nucléotidique modifiée

SEQ ID n° : 4

ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG 36

CTG CCC CTC TTT GAG CCC AAG, 57 .

3. Gène d'une Taq polymérase dans lequel la séquence des trente premières bases nucléotidiques du gène natif qui code pour les dix premiers acides aminés de la protéine mature native, a été changée en substituant par conséquent la séquence nucléotidique modifiée

SEQ ID N° : 2

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG 33 .

4. Gène selon l'une quelconque des revendications 1 à 3, possédant un site de restriction adjacent au codon d'initiation et situé en amont de celui-ci (ATG), et le même site de restriction adjacent au codon stop (TGA) et en aval de celui-ci.

5. Gène selon la revendication 4, dans lequel les sites de restriction sont codés par la séquence nucléotidique GAATTC.

6. Gène selon la revendication 3, dans lequel la séquence native :

SEQ ID N° : 1

ATG AGG GGG ATG CTG CCC CTC TTT GAG CCC AAG 33

est altérée en

SEQ ID N° : 2

ATG CGT GGT ATC CTG CCT CTG TTT GAG CCG AAG 33 .

7. ADN polymérase thermostable de Thermus aquaticus codé par le gène selon la revendication 1 ou la revendication 2, possédant comme séquence des premiers acides aminés de la protéine nature :

SEQ ID N° : 9

Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys .

1

5

8. Procédé d'augmentation de la production d'une Taq polymérase comportant les étapes consistant à :

- A) fournir un vecteur ayant le gène selon l'une quelconque des revendications 1 à 3 ;
 B) transfecter un hôte d'E. coli compatible à l'aide du vecteur de A), obtenant de ce fait des cellules hôtes d'E. coli transformées ; et
 C) cultiver les cellules transformées de B) dans des conditions de croissance produisant de ce fait une Taq polymérase synthétisée par les cellules hôtes transformées.

9. Procédé selon la revendication 8 dans lequel le vecteur de l'étape A possède un promoteur inductible.

10. Procédé selon les revendications 8 ou 9, dans lequel la production d'une Taq polymérase est induite par l'isopropyl- β -D-thiogalactosidase (IPTG).

11. Vecteur ayant le gène selon l'une quelconque des revendications 1 à 3, ledit vecteur possédant :

- i) des marqueurs pouvant être sélectionnés,
 ii) un promoteur approprié, et
 iii) des séquences de régulation correctes afin de commander une expression génique.

12. Cellule hôte d'E. coli comportant le vecteur selon la revendication 11.

Patentansprüche

1. Gen für Taq-Polymerase, bei dem die Sequenz der ersten dreißig Nucleotidbasen in dem nativen Gen, die die ersten zehn Aminosäuren in dem reifen nativen Protein kodieren durch Insertion der Sequenz:

SEQ ID NO:8

GAC TAC AAG GAC GAC GAT GAC AAG

24.

zwischen dem Startcodon (ATG) des reifen nativen Proteins und dem Codon (AGG) für die zweite Aminosäure des reifen nativen Proteins geändert worden ist.

2. Gen für Taq-Polymerase, bei dem die Sequenz der ersten dreißig Nucleotidbasen in dem nativen Gen, die die ersten zehn Aminosäuren in dem reifen nativen Protein kodieren, durch Substitution mit der modifizierten Nucleotidsequenz:

SEQ ID NO:4

ATG GAC TAC AAG GAC GAC GAT GAC AAG CGT GGT ATG

36

CTG CCC CTC TTT GAG CCC AAG,

57.

geändert worden ist.

3. Gen für Taq-Polymerase, bei dem die Sequenz der ersten dreißig Nucleotidbasen in dem nativen Gen, die die ersten zehn Aminosäuren in dem reifen nativen Protein kodieren, durch Substitution mit der modifizierten Nucleotidsequenz:

SEQ ID NO:2

ATG CGT GGT ATG CTG CCT CTG TTT GAG CCG AAG

33.

geändert worden ist.

4. Gen nach einem der Ansprüche 1 bis 3, das eine Restriktionsstelle stromaufwärts von dem Startcodon (ATG) und diesem benachbart aufweist und dieselbe Restriktionsstelle stromabwärts von dem Stopcodon (TGA) und diesem benachbart aufweist.

5. Gen nach Anspruch 4, bei dem die Restriktionsstellen durch die Nucleotidsequenz GAATTC kodiert werden.

6. Gen nach Anspruch 3, bei dem die native Sequenz:

SEQ ID NO:1

ATG AGG GGG ATG CTG CCC CTC TTT GAG CCC AAG

33

geändert ist zu der

SEQ ID NO:2

ATG CGT GGT ATC CTG CCT CTG TTT GAG CCG AAG

33.

7. Thermostabile Thermus aquaticus-DNA-Polymerase, die durch das Gen von Anspruch 1 oder Anspruch 2 kodiert wird, die als die erste Aminosäuresequenz in dem reifen Protein

SEQ ID NO: 9

Met-Asp-Tyr-Lys-Asp-Asp-Asp-Lys.

1

5

aufweist.

8. Verfahren zum Steigern der Produktion von Taq-Polymerase, umfassend die Schritte:

- A) Bereitstellen eines Vektors mit dem Gen von einem der Ansprüche 1 bis 3;
- B) Transfection eines kompatiblen E. coli-Wirtes mit dem Vektor von A), wobei transformierte E. coli-Wirtszellen erhalten werden; und
- C) Kultivieren der transformierten Zellen von B) unter Wachstumsbedingungen, wodurch Taq-Polymerase erzeugt wird, die von den transformierten Wirtszellen synthetisiert wird.

9. Verfahren nach Anspruch 8, bei dem der Vektor von Schritt A einen induzierbaren Promotor aufweist.

10. Verfahren nach Anspruch 8 oder Anspruch 9, bei dem die Produktion von Taq-Polymerase mit Isopropyl-β-D- thio- galactosidase (IPTG) induziert wird.

11. Vektor mit dem Gen von einem der Ansprüche 1 bis 3, wobei der Vektor aufweist:

- i) auswählbare Marker,
- ii) einen geeigneten Promotor, und
- iii) geeignete Regulatorsequenzen zum Kontrollieren der Genexpression.

12. E. coli-Wirtszelle, die den Vektor von Anspruch 11 umfaßt.

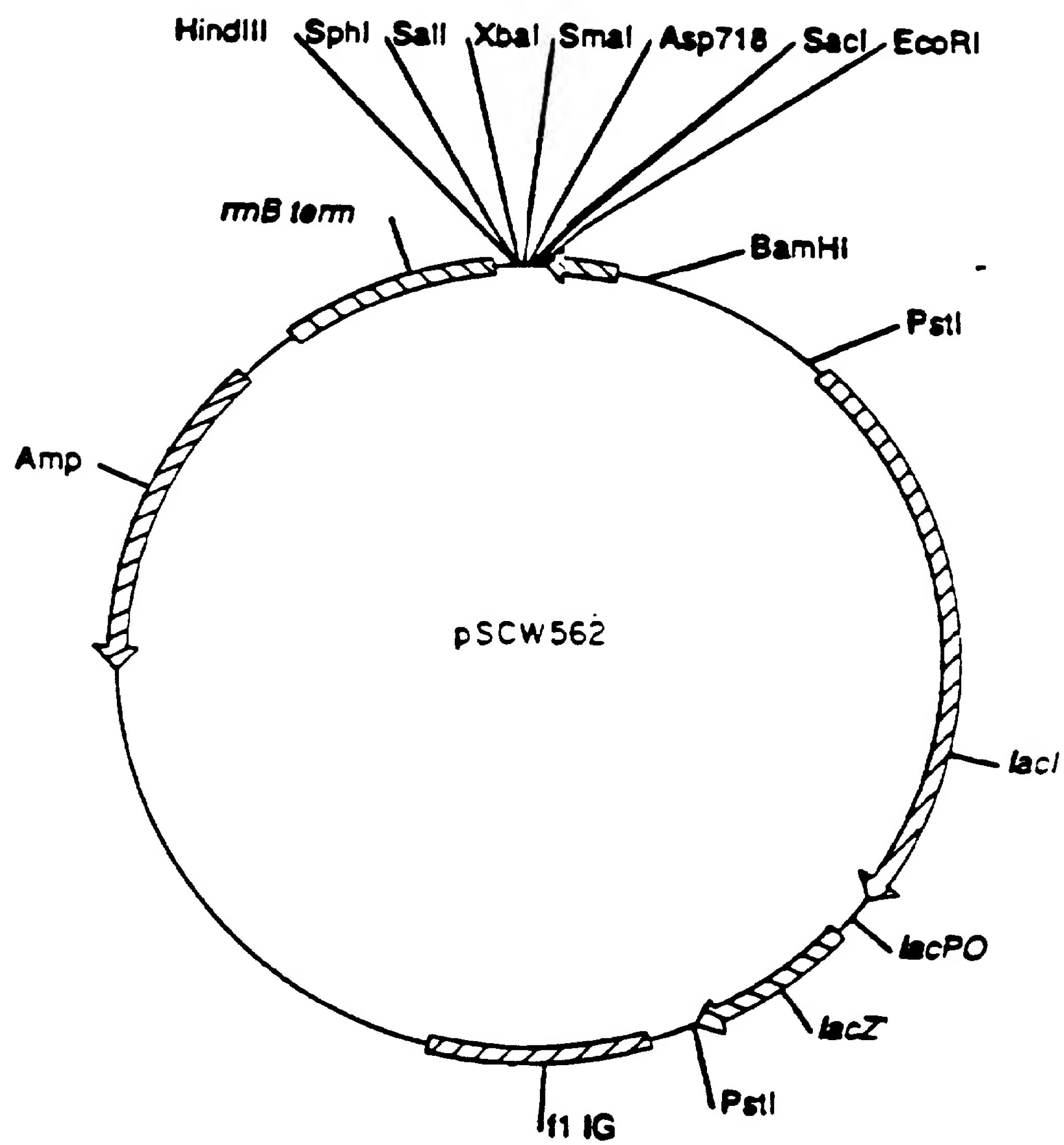


FIG. 1